THE SUSCEPTIBILITY OF THE CHORIO-ALLANTOIC MEMBRANE OF CHICK EMBRYOS TO INFECTION WITH THE FOWL-POX VIRUS *

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For studying a representative of the pox group of virus diseases, fowl-pox has many advantages, among them being the fact that infectious material is readily obtained and easily handled, since the disease is limited to fowls. It is believed that knowledge gained concerning this disease may be serviceable in the study of other members of the pox group. Hence, this virus has been the subject of several problems investigated in this laboratory during the last three years.

Fowl-pox, like the other pox diseases, is characterized by the appearance of eruptive skin lesions. The spontaneous fowl-pox nodules appear especially on the unfeathered parts of chickens, although experimental lesions may be easily induced in specialized epidermal structures such as cornea, feather follicles and oil gland. The lesions consist of a hyperplasia of the epithelial cells, with inclusion bodies in their cytoplasm. It has been shown that these inclusions are composed of groups or colonies of minute (Borrel) bodies.[1] While the presence of inclusions has always been considered pathognomonic of the disease, recent experimental work has given much evidence in favor of the theory that the Borrel body, one component of the inclusion, is the etiological agent of the disease.[2, 3, 4]

Heretofore, fowl-pox has been studied only in the grown or newly hatched chicks, or in tissue culture. Tissue culture experiments with this virus have, however, been few and inconclusive.[5, 6] The present paper deals with the inoculation of the virus into embryonic tissues in the incubating egg.

The chorio-allantoic membrane of the chick embryo has been used by a number of investigators for the study of the growth of various implanted tissues. Rous and Murphy [7] were the first to use

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this technique for the study of tumors. Danchakoff [8] has used the method to grow embryonic chick tissues. Since the publication of these two papers the technique has been used frequently in experiments with auto- and heteroplastic grafts, as well as in those with auto- and heterogeneous tumors. The production of experimental infection in the chorio-allantoic membrane has, however, been done only in the one instance where Rous and Murphy grew the virus of the Rous sarcoma.[7] *

Technique for Obtaining Sterile Virus

One of the most important steps in the technique of virus inoculations of embryonic chick membrane is the use of material free from contaminating microörganisms. The only methods available in the past for obtaining uncontaminated fowl-pox virus have involved considerable dilution of it, such as filtering through a Berkefeld candle or using the virus which capillary attraction has caused to rise higher than bacteria on a piece of filter paper.[9] The virus thus obtained is associated with the minute Borrel bodies in suspension. It was thought that some method which would provide uncontaminated virus consisting largely of inclusions would be especially useful. A number of such methods have been developed recently in connection with this work. Since each may prove advantageous for certain types of experiments, these will be described in detail before continuing with the experiments on embryo inoculation.

Method I. Uncontaminated virus can be obtained directly from fowl-pox nodules on the skin of a chick. The following technique proved to be the most satisfactory. After plucking the feathers from the heads of young chicks, 1 to 2 weeks old, virus was inoculated at three points about 1 cm. apart, to allow the development of separate nodules which could be removed by one stroke of the knife. Since nodules of more than seven days' development are likely to be invaded by pyogenic bacteria, the chick was sacrificed six or seven days after inoculation. The head was bathed with 95 per cent alcohol and allowed to dry. With a sterile cataract knife, a nodule was cut off rapidly at a level deep enough to obtain the

* Mention is made by Askanazy[10] of the production of tuberculous chicks by the infection of fertile eggs.

infected cores of most of the follicles. The severed nodule was placed epithelial surface down on a sterile glass slide, while, with a pair of fine curved forceps, the infected cores were forced out of the follicles from the cut surface. These small pieces were washed twice with sterile Tyrode's solution and stored at 4° C in a small amount of the same solution. One piece was tested in glucose yeast broth. If no bacterial growth was apparent within twenty-four hours, the remaining virus was made into a suspension for inoculation by grinding with a few drops of Tyrode's solution.

METHOD II. A second method for obtaining uncontaminated inclusions, and one which is especially useful for tissue culture experiments, was developed during work with the inoculation of single inclusions picked out with the Chambers microdissection apparatus.[2] For this work inclusions from lesions of seven to ten days' development were used. The tissue was digested with 1 per cent trypsin to free the inclusions. These were then carefully washed several times with sterile saline. Finally a single inclusion was picked up with a minute sterile pipette and deposited on a sterile cover slip. Though only a few such experiments were tried, plasma cultures with inclusions thus washed remained sterile in every instance. However, unless single inclusions are required, the first method is preferable because it is much less difficult and affords a larger amount of virus.

METHOD III. A third method which is useful when numbers of free inclusions are desired was developed in the course of experiments on the effect of 1 per cent potassium hydroxide on the virus.[4] Since the usual bacteria are destroyed after a few hours in 1 per cent potassium hydroxide, while fowl-pox virus, in the form of inclusion bodies, survives for at least twenty-four hours, we performed a number of experiments to see if virus free from contaminating microörganisms could actually be obtained after one day's treatment with potassium hydroxide. It was found that in highly contaminated pieces of fowl-pox tissue certain moulds, and occasionally a bacillus, persisted for three or more days in 1 per cent potassium hydroxide. After such a long period of treatment the strength of the virus is much diminished and may be completely destroyed. By using inclusions, freed from the tissue by tryptic digestion and carefully washed several times in sterile saline, the number of contaminating organisms was greatly diminished as shown by inoculation of

the material into glucose agar, and plating. Proceeding with sterile precautions throughout, it was found that after twenty-four hours in 1 per cent potassium hydroxide, agar plates containing 10 cc. of agar and 1 cc. of a suspension of the treated inclusion bodies usually showed no contamination. Occasionally, however, one or two colonies of a mould persisted. Inclusions freed to this degree from contaminating organisms could then be used for most types of experimental work, though they were less satisfactory than inclusions obtained by the two preceding methods.

METHOD IV. The fourth method for obtaining uncontaminated virus came as a natural development of the successful inoculation of embryonic chick membranes and will be described later. Methods I and IV provide for the production of virus which has never been in contact with bacteria, a fact which should make these virus preparations valuable in immunological experiments.

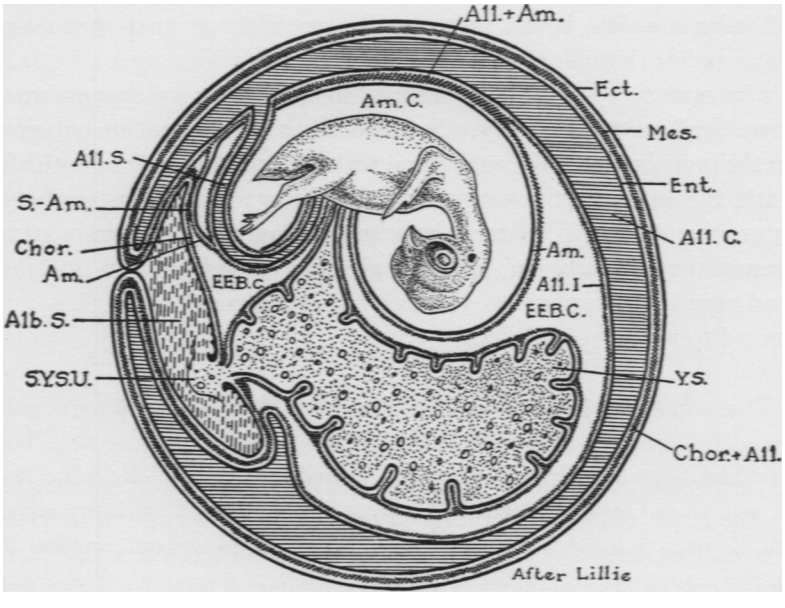
TECHNIQUE FOR INOCULATION OF CHICK EMBRYOS

The technique for opening the eggs used in our experiments was based on that described by Clark.[10] We omitted the use of a hot box, but kept the air sac immersed in water at 39° C. Keeping the air sac thus immersed prevented sagging of the egg contents when the egg was opened. We found that a piece of plasticene molded to fit the egg was a convenient support. The top surface of the egg was sterilized by bathing in alcohol and flaming. Then, proceeding with sterile precautions, a window, 7 to 10 mm. square was made by cutting or scraping with a sharp point. We found the sharp end of a scissors blade very convenient for this. After the shell was removed, the shell membrane was cut away carefully in order to expose the chorio-allantoic membrane.

For purposes of clarity in the description of the inoculation of chick embryos and the chorio-allantoic membrane, a diagram of the 12 day chick with its membranes has been reproduced from Lillie [11] (Text-Fig. 1). One label designating the chorio-allantoic membrane (the membrane formed by the fusion of chorion or serosa with the outer wall of the allantoic sac), has been added to the original diagram.

Two sorts of inoculation were attempted. The simpler procedure consisted in slightly injuring the chorio-allantoic membrane by

pricking with a needle and applying a drop of an uncontaminated virus suspension (Method I) to the injured area. In the second and more difficult operation, the skin of the embryo itself was inoculated. This involved cutting the chorio-allantoic membrane and amnion and slightly abrading the skin of the embryo, since some injury to epithelial cells favors the invasion of the virus.



TEXT-FIG. 1

Alb. S., albumin-sac. All. I., inner wall of the allantois. All. C., cavity of allantois. All. S., stalk of allantois. All. + Am., fusion of allantois and amnion. Chor. + All., fusion of chorion and outer wall of allantois. Am., amnion. Am. C., amniotic cavity. Chor., chorion. Ect., ectoderm. E.E.B.C., extra-embryonic body cavity. Ent., entoderm. Mes., mesoderm. S.-Am., sero-amniotic connection. S.Y.S.U., sac of the yolk-sac umbilicus. Y.S., yolk-sac.

In most of the techniques described in the literature the original piece of shell is replaced following the operative procedure and paraffined, so that the egg can be turned daily to continue normal development. Since we desired to watch the effects of the virus and to get sections at once if the chick should die, we substituted a glass cover slip for the original shell, fixing it upon a ring of vaseline, and returned the egg to the incubator immediately after the operation. This technique necessitated keeping the window uppermost during

the rest of embryonic growth. The lack of turning caused usually an oval depression and fold in the membrane directly below the opening. No other abnormality due to lack of turning appeared to occur, for a number of chicks hatched normally from eggs which had been subjected to this treatment.

Embryos at various stages of development were used. Since it takes about four days for a well defined fowl-pox lesion to appear after inoculation, it was necessary to inoculate at least that many days before hatching. The most extensive lesions were obtained six to seven days after inoculation, so that 10 to 15 day embryos were used most frequently. Occasionally, a contamination occurred in the inoculation of the egg. Sometimes a mould grew symbiotically with the virus in the embryonic membrane. Such contaminated eggs were discarded. Except as a contaminating organism is introduced upon inoculation, the eggs are relatively free from infection and remain, according to Rettger,[12] a sterile medium, unless subjected to moisture and dirt.

Results of Inoculation of Embryo Chick and Membranes

Fowl-pox infection of the chorio-allantoic membrane occurred as the result of inoculation in every case where the embryo survived for at least four days. Infections were first noted when thickened areas on the chorio-allantoic membrane were detected after several days' incubation. That a fowl-pox infection was definitely present was proved by three tests. The tissue, removed with sterile precautions and inoculated onto the scarified epithelium of adult hens, produced a massive fowl-pox lesion. Smears of the lesions, stained by Morosow's method,[13] showed Borrel bodies present in great numbers. Histological sections of the tissue showed the typical picture of the fowl-pox lesion (Figs. 2 and 9). These lesions are characterized by a marked hyperplasia of the ectodermal layer and an accompanying thickening of mesoderm as well. (Compare with normal membrane (Fig. 1).) Frequently hyperplasia of the entoderm occurs also. In the cells of the ectodermal layer many large inclusions are present, while in the entodermal layer, when occasionally a definite infection is present, inclusions are few and small. The lack of an inflammatory exudate, even in an advanced stage of the infection, should be noted.

In order to show the gross appearance of the infected areas several infected eggs were fixed in Zenker's fluid with the membranes intact. Sometimes the infection occurred in just a few areas, presumably at the site of the original inoculation (Fig. 6), but more often the infected area covered half the surface of the serosa (Fig. 5), and frequently small isolated areas of infection were found at a distance from the large primary lesion.

Upon the discovery that the outer embryonic membrane always developed this large area of infection directly below the window in the egg, it was decided to attempt the removal, with sterile precautions, of pieces of the infected membrane. By flaming the whole egg and carefully removing the coverslip, the infected tissue was exposed. Pieces of the infected membrane were cut away and washed in sterile Tyrode's solution. A sample of the tissue was inoculated into glucose yeast broth, and it was found that uncontaminated material could be obtained readily in most cases. The infected tissue in Tyrode's solution was then stored at 4° C until needed. Virus thus prepared was used generally within two weeks, although samples stored for several months were shown to be still virulent. Uncontaminated virus was obtained by this fourth method in much larger quantities than any other means so far devised. Consequently this method was used in subsequent experiments where such virus was required. The virus obtained by Method IV is convenient for almost any type of fowl-pox problem, if uncontaminated virus is needed, for the material can be used in a number of ways — as bits of infected tissue, as free inclusions which can be teased out from it, or as a Borrel body suspension, made by grinding the tissue with saline.

The inoculation of the embryonic skin caused considerably more trauma than the inoculation of the chorio-allantoic membrane. The percentage mortality was so great that this operation was soon abandoned, since the membrane inoculations proved very satisfactory. Several successful embryo inoculations were made, however. One infection of an embryo foot was produced (Fig. 7), and other infections were obtained, notably at the umbilicus. Although it was not intended to inoculate at this point, injury to the umbilical region must have occurred during the operation, for, with the inoculation of chorio-allantoic membrane alone, umbilical lesions were not obtained except on chicks which hatched and survived for several days.

Concerning the effect of fowl-pox on embryonic development and ability to hatch, our information is scant since most of the embryos were sacrificed before hatching. A few chicks, however, were hatched from eggs with infected membranes. These chicks were apparently normal, though they must have carried the virus, since all of those that were not sacrificed immediately developed fowl-pox lesions six to eight days later. The nodules appeared most frequently at the base of the beak or about the umbilicus. This infection may have been the result of autoinoculation during the process of pecking through the shell and escaping from the infected membranes. A second possibility is that the cells at the beak and umbilicus, injured during hatching, were infected by virus in the blood stream. The extreme vascularity of the chorio-allantoic membrane would make it seem highly improbable that the blood would remain virus free. Proof of the presence of virus in the circulation was obtained from a series of experiments in which pieces of liver were removed with sterile precautions from chicks which had either hatched from or died in eggs with infected membranes. In the majority of cases where liver material was inoculated onto the scarified epithelium of chicks a small lesion was obtained. In one case out of six, the inoculation of liver material produced no lesion. The lack of massive lesions as a result of these inoculations seemed to us to indicate that though the virus is present in small quantities in the blood stream, it is not actively proliferating there. This observation is in accordance with an accepted view concerning the virus present in the circulation of infected adult hens.[14]

Following the successful inoculation of the membranes of 10 to 15 day embryos, the question arose as to how early in the development of the embryo a successful inoculation could be made. Danchakoff [8] working with embryonic grafts on the allantois states that embryos younger than 7 days could not be used because of the small size of the allantois at that stage. Whether or not the absence of the chorio-allantoic membrane made our inoculations of membranes of embryos younger than 6 days more difficult, it was found that such inoculations were not generally successful. The chorio-allantoic membrane of 6 day embryos was infected with no difficulty, and on one occasion we succeeded in infecting this membrane in an embryo which was inoculated at the 4 day stage (Fig. 8). In younger embryos, the injury caused by opening the egg and pricking the membranes seemed to be greater, for the embryos usually died too soon

after inoculation for a lesion to develop. The technical difficulties involved caused us to abandon an attempt to determine the susceptibility of embryos of less than 4 days' development. It is not intended, therefore, to imply that younger embryos could not be infected.

Detailed study of a number of histological sections of infected chorio-allantoic membrane revealed the fact that entodermal as well as ectodermal epithelium could be infected. Inclusion bodies were usually less numerous, and hyperplasia was less marked than in infected ectoderm (Figs. 9, 10 and 11). Entoderm seems to be much less susceptible than ectoderm since entodermal infection did not occur in every case of successful ectodermal infection. A somewhat similar retarded response of entoderm was found by Huxley and Murray in work with chorio-allantoic grafts.[15] The stimulus in this case, however, was an operative one rather than one caused by an infection.

It should be mentioned in passing that occasionally the membrane was torn at inoculation, and at this point infected ectodermal cells had fused with cells of the entodermal layer. For the identification of a true entodermal infection, however, we were able to obtain sections of isolated nodules at a distance from the point of inoculation, where there was no possibility of ectodermal cells being present in the entodermal layer.

A further indication that entoderm is less susceptible than ectoderm to fowl-pox infection is seen in the fact that entodermal derivatives of the adult hen are rarely infected. Instances of spontaneous lesions in the crop have occasionally been observed. Two instances of spontaneous lesions of the trachea have also been seen. These lesions were not isolated nodules, but part of massive lesions of the throat. Though the infected areas appeared to be in columnar epithelium, the lesions were not considered to be complete proof of the susceptibility of tracheal epithelium (*i.e.* epithelium of entodermal origin), since they were not isolated from epithelium of ectodermal derivation. Using uncontaminated virus (Method IV), an attempt to corroborate the experimental infection of embryonic entoderm by the production of a fowl-pox lesion in adult tracheas was made.

With sterile precautions, the tracheas of two hens were cut, scarified and inoculated. The hens were sacrificed eleven and thirteen days respectively after inoculation. In both cases a gross infection

of the skin of the neck occurred but there were no adhesions to the trachea. In each experiment the mucous membrane of the trachea contained a number of small nodules, sections of which showed the typical fowl-pox lesion (Figs. 12 and 13). The infection of entodermal tissues, both adult and embryonic, is thus shown to be possible under experimental conditions. Uncontaminated virus may prove useful in other experiments of this type. Using such virus, intracerebral inoculations might prove interesting, as well as further inoculations of entodermal derivatives such as the mucous membrane of the intestine, or of mesodermal epithelium, *e.g.*, that in the kidney.

The position of the capillaries in some of the sections of infected chorio-allantoic membrane corroborates Danchakoff's theory concerning the development of the respiratory net of the allantois.[16] The allantois is both the respiratory and excretory organ of the embryo. By the thirteenth to fifteenth day its capillary network has in some manner become the outermost layer of living cells. This is contrary to the usual belief that the mesodermal cells of the embryo must always be bounded by two germ layers. This phenomenon was explained by Füllborn [17] as being due to the degeneration of the epithelial cells of the chorion. Danchakoff, however, holds that the final position of the capillary net is due to a migration of the capillaries. She proved that ectodermal cells were still present, by subjecting them to the pressure of grafted tissue, after which keratinization occurred.

Our sections indicate that migration of capillaries rather than degeneration of epithelial cells has occurred in the change of position of the respiratory net. The ectodermal layer can be distinguished because of the infected epithelium in at least part of the section. If the fowl-pox lesion had developed before the migration of the capillary net, this migration was prevented either wholly or partially. Some sections (Fig. 2) show the capillary net entirely below the ectoderm in the heavily infected area, while it occupies a midplace in the ectoderm of the same lesion where the infection is less (Fig. 3), and is found on the surface with ectodermal cells below when it reaches a non-infected area (Fig. 4).

In a number of the sections of infected serosa, epithelial pearls were noted, suggesting the possibility that rapid passage of the infection from one embryo to another might result in massive hyper-

plasia resembling an epithelial tumor. Accordingly a series of experiments was begun in which it was attempted to graft bits of infected tissue from the chorio-allantoic membrane on normal membranes. After a period of four to seven days, the original explant plus the newly infected area surrounding it was removed. Part of the block was used for sections and part for transplantation. The explants varied in size from 0.5 to 1.5 c.mm. It was found that identification of the transplant in the gross was difficult, due to its inclusion in the fresh growth of infected tissue. By dipping the transplants in a suspension of India ink in saline, however, enough carbon adhered to the tissue so that the transplant could be identified even after it had produced a heavy infection in the host membrane. Since it was thought that the ink might injure the cells of the transplant, both plain and inked transplants were tried, and several of each type were identified in sections. The series was terminated with the third transplant. Study of the sections obtained showed that in neither inked nor plain transplants had a true graft occurred. Apparently the infected cells degenerated too rapidly for them to become established in the new location. Though hyperplasia was evident, the type of lesion in the second and third transplants was not different from that obtained after the first inoculation. It was concluded that the epithelial pearls were probably due to mechanical displacement of the epithelium in the membrane.

Discussion

Experiments upon fowls have shown the great susceptibility of ectodermal cells to infection with the virus of fowl-pox. When the virus is injected intravenously the resulting lesions are almost entirely confined to the skin. Sometimes, however, the epithelium of the esophagus, crop and trachea are affected, showing the ability of the virus to multiply in epithelium having an entodermal derivation. From the fact of the infrequent occurrence of spontaneous gastro-intestinal and tracheal lesions, and from the characteristics of these lesions whether spontaneous or experimental, it seems evident that the virus of fowl-pox affects ectodermal epithelium much more readily than entodermal, and increases more abundantly in the former. In common with certain other cytotropic viruses, that of fowl-pox seems thus to possess a high degree of cellular specificity.

The experiments recorded in this paper show that the same specificity obtains when this virus is brought into contact with the tissues of chick embryos, and in similar degree. That is to say, in the embryo as in the adult fowl, ectodermal squamous epithelium is more susceptible, while entoderm of the allantois is less readily affected, and in the latter cells, virus regenerates much less abundantly, judging from the number and size of cellular inclusions.

This susceptibility appears very early in the cellular differentiation of the embryo. With the technical methods at our disposal it was not determined how early the embryonic cells acquire this susceptibility, or, to state it differently, how soon certain embryonic cells lose their susceptibility, supposing that the earliest undifferentiated cells from the ovum are all susceptible to the virus. It would be of great interest to know whether or not ectodermal and entodermal epithelium acquire their susceptibility as a result of cellular differentiation.

By the use of the chorio-allantoic membrane of chick embryos for the production of the infection, the preparation of non-contaminated concentrated virus in fairly large quantities is made possible. This virus, being the infected tissue grown in and obtained from a sterile medium, can thus be used in whatever form desired, *i.e.*, as infected tissue, inclusion bodies, or a suspension of Borrel bodies. This method and also Method I, described in this paper, have an advantage over any other known preparations of non-contaminated fowl-pox virus, in that, since the cells in which the virus has developed have never been contaminated, the virus should be free from antigens not directly associated with the disease. This fact should make it especially useful in immunological experiments.

One of the uses of such a virus preparation has been demonstrated in the successful inoculation of adult chicken trachea with fowl-pox virus. Inoculations of such virus into the internal organs of the chicken, especially those with epithelial surfaces such as the kidney, might give valuable information.

The use of embryonic chick membranes as a medium for the production of other virus infections, *e.g.*, vaccinia, might prove advantageous in the study of the etiology and development of these diseases.

SUMMARY

1. Ectodermal and entodermal cells of the chorio-allantoic membrane of the chick, as well as embryonic chick skin, are susceptible to infection with the virus of fowl-pox at an early stage in the development of the embryo. Whether or not this specific susceptibility is acquired as a result of cellular differentiation has not been determined.

2. Four methods for the isolation of uncontaminated fowl-pox virus are described.

3. In two of these methods the virus is developed in tissue that has never been contaminated by extraneous microörganisms.

4. Fowl-pox infection in the trachea of the adult hen has been induced by means of inoculation with uncontaminated virus.

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DESCRIPTION OF PLATES

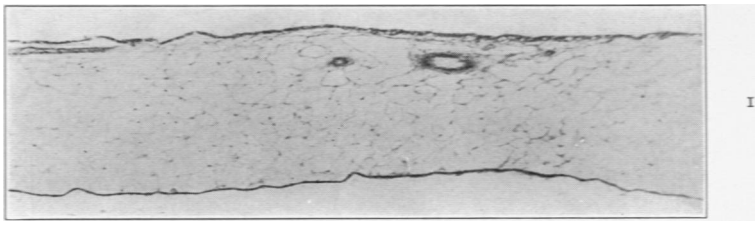
PLATE 40

FIG. 1. Normal chorio-allantoic membrane from hatching chick. × 50.

FIG. 2. Ectoderm of chorio-allantoic membrane of chick embryo showing fowl-pox infection, and the position of the capillary net below a heavily infected area. Section taken five days after inoculation. Note absence of inflammatory exudate. × 200.

FIG. 3. An intermediate position of capillary net in the hyperplastic ectodermal epithelium adjacent to massive fowl-pox infection of embryonic membrane (Fig. 2). × 200.

FIG. 4. Position of capillaries on surface of non-infected ectoderm adjacent to area (Fig. 3) of hyperplastic epithelium in fowl-pox infected membrane. × 200.



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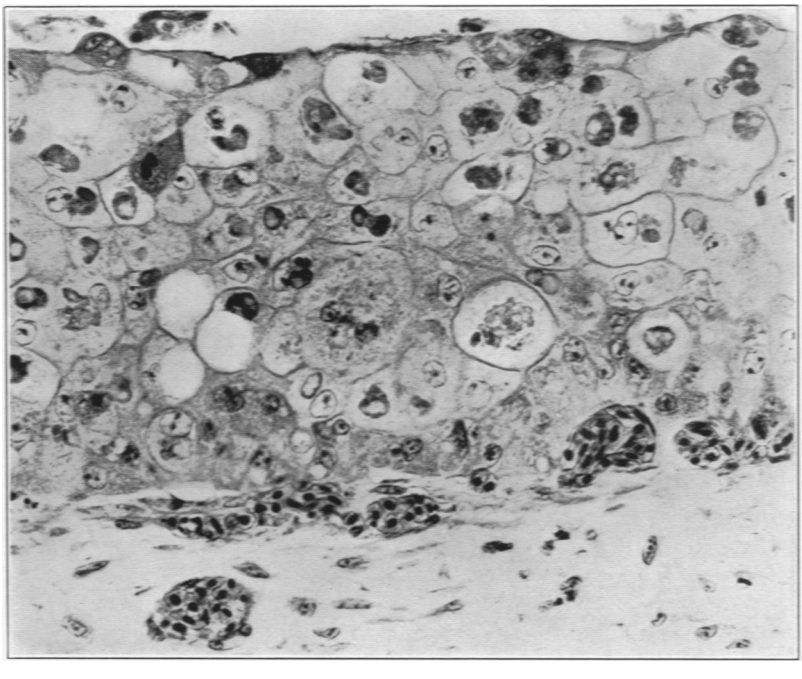

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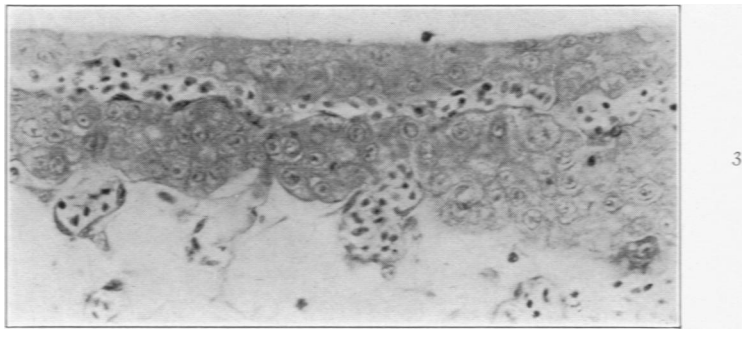

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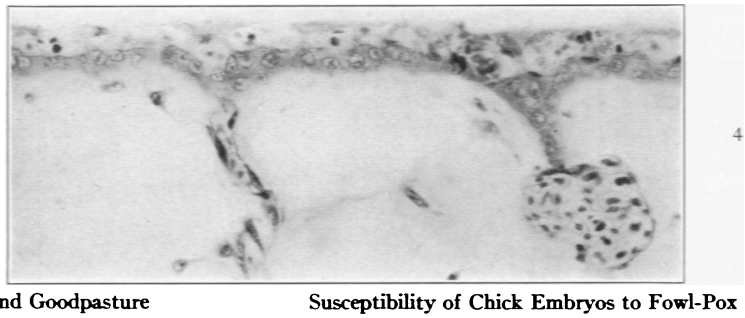

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PLATE 41

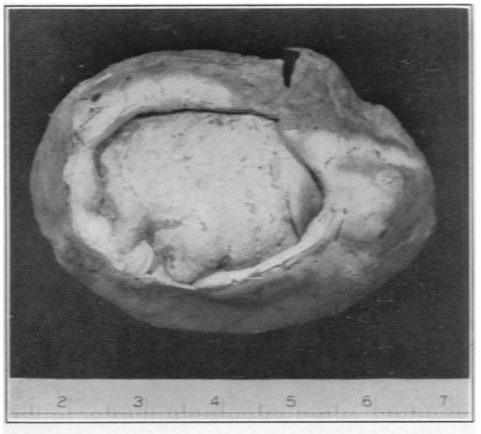
FIG. 5. Massive fowl-pox infection in chorio-allantoic membrane of 15 day embryo, seven days after inoculation.

FIG. 6. Isolated areas of fowl-pox infection in chorio-allantoic membrane of 16 day embryo, seven days after inoculation (shown at right).

FIG. 7. Fowl-pox infection in epithelium of foot of 21 day embryo, seven days after inoculation. × 50.

FIG. 8. Fowl-pox infection in ectoderm of chorio-allantoic membrane resulting from inoculation at 4 day stage. × 50.

FIG. 9. Fowl-pox infection in chorio-allantoic membrane showing massive lesion in ectoderm and hyperplasia of entoderm. × 50.



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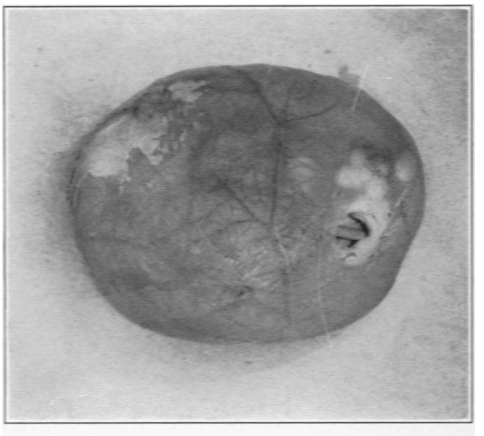

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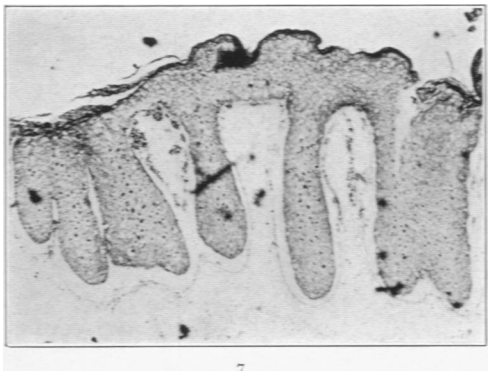

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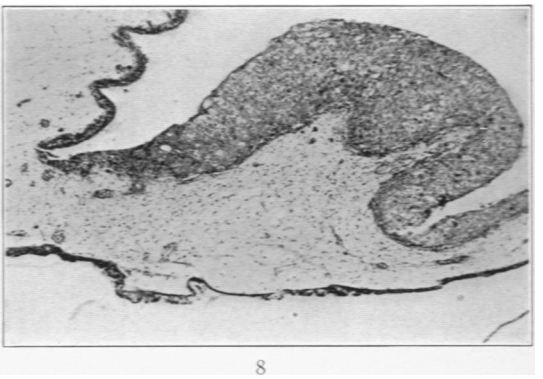

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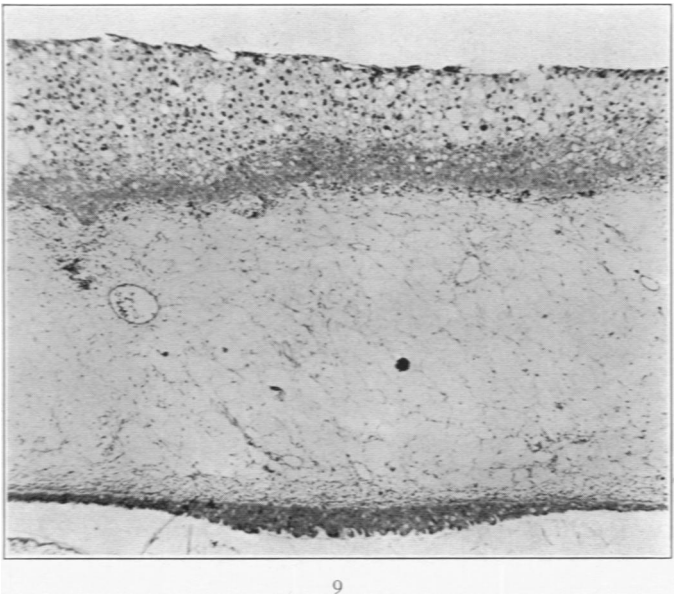


9

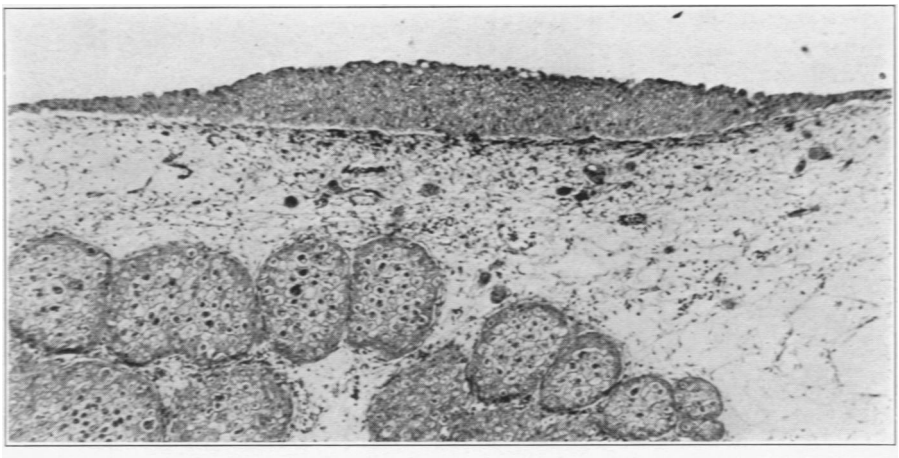
PLATE 42

FIG. 10. Isolated area of fowl-pox infection in entoderm of chorio-allantoic membrane, six days after inoculation. Note small size of inclusions and cells as compared with those of epithelial nodules from ectodermal layer in lower portion of picture. × 50.

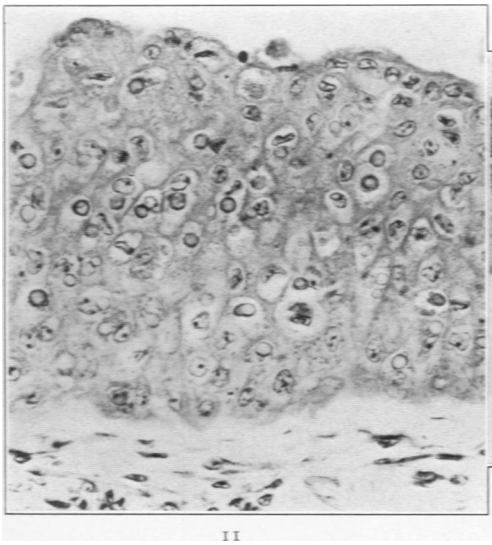
FIG. 11. A portion of Fig. 10 under higher magnification, showing fowl-pox infection in entoderm of chorio-allantoic membrane. Note that inclusions are smaller and hyperplasia less than in infected ectoderm of Fig. 2. × 200.

FIG. 12. Experimental fowl-pox infection of mucous membrane of trachea. *Cf.* normal epithelium at left with infected area at right. × 50.

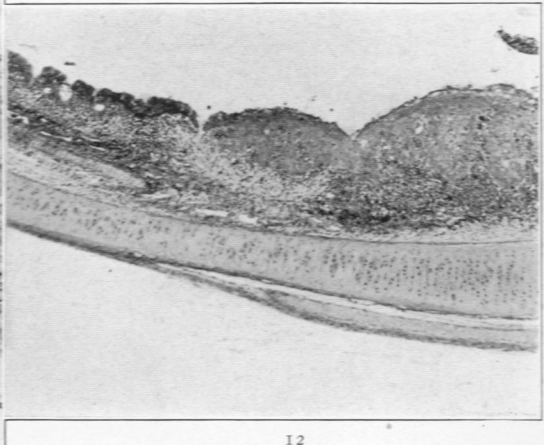
FIG. 13. A portion of Fig. 12 under higher magnification, showing experimental fowl-pox infection of mucous membrane of trachea. Note metaplasia of epithelium and relatively few inclusions. × 200.



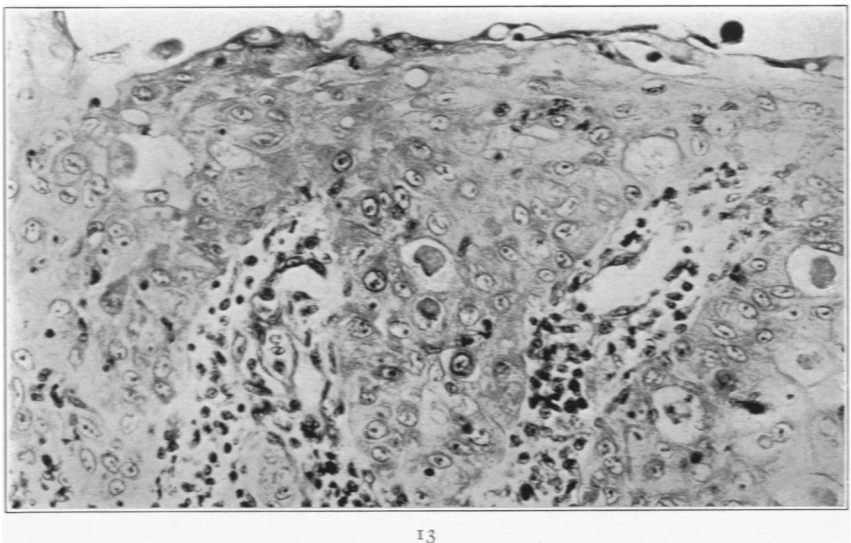
10



11



12



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